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PRINCIPAL INVESTIGATOR: Dr. Robert W. Veltri

CONTRACTING ORGANIZATION: Johns Hopkins University
Baltimore, MD 21218-2680

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ROBERT W. VELTRI

E-Mail: rveltri1@jhmi.edu

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14. ABSTRACT

The Cancer/Testis Antigens (CTAs) are a group of proteins normally confined to germ cells but aberrantly expressed in several cancers. The central hypothesis of this grant application is that a CTA-based biomarker can be used to discern LPCa from MPCa. In the first year of this grant, we determined gene expression of 22 candidate CTAs by Nanostring and validated by qRT-PCR. During the second year of the grant, we used ROC curve analysis and identified 8 CTA genes (CEP55, NUF2, PAGE4, PBK, RQCD1, SPAG4, SSX2 and TTK), which expression pattern is significant different between aggressive and indolent tumors. For the third year of the grant, we evaluated the gene expression of these 8 CTAs in PCa and benign adjacent paired tissues from 24 patients. The only CTAs differentially expressed between non-cancer and cancer areas were PAGE4, SPAG4 and SSX2. For all the selected biomarker candidates, we obtained commercial antibodies from two sources and performed optimization using training TMAs containing normal and tumor prostate tissue. Quantification of the CTAs protein expression is being performed using an automated image system. We also performed CTA expression analysis in PCa cell lines (DU145, LNCAP, PC3, PC3 Epi, PC3 EMT and BPH1) by qRT-PCR and Western Blot. Absence of gene expression correlated

15. SUBJECT TERMS
Prostate cancer; metastatic prostate cancer; cancer/testis antigens (CTA); biomarker; gene expression; nCounter; Nanostring; qRT-PCR

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TABLE OF CONTENTS

1. INTRODUCTION

Prostate cancer (PCa) is the most commonly diagnosed cancer and the second leading cause of cancer-related deaths among men in the US (Siegel et al., 2014). The introduction of the prostate specific antigen (PSA) test has greatly aided to the early detection of PCa. Detectable levels of PSA are the earliest sign of recurrent disease after radical prostatectomy (RP) (Pound et al., 1999). Besides its sensitiveness, it is estimated that 23-44% of patients submitted to RP will progress with detectable PSA levels and will never present recurrence (Draisma et al., 2009). Thus, a clinical dilemma today in the management of PCa is to distinguish men with aggressive disease who need definitive treatment from men with indolent disease not requiring immediate intervention. As PSA screening is not capable of discriminating between low risk and aggressive PCa the identification of novel biomarkers is critical to offer patients adequate treatment following RP. The central hypothesis of this study is that a CTA-based biomarker can be used to discern PCa patients with aggressive disease and hence would need definitive treatment from those in whom it is less likely to recur and would not require immediate intervention. The Cancer/Testis Antigens (CTAs) are a unique group of heterogeneous proteins that are normally confined to germ cells in normal testis and placenta, but aberrantly expressed in several types of cancers (Scanlan et al, 2004). Unfortunately, their potential as biomarkers in PCa has not been rigorously explored and a coordinated expression pattern of the CTAs associated with tumor grade/stage has not been demonstrated to date for any type of cancer. This hypothesis will be addressed with the following specific aims: 1) to identify candidate CTAs that are differentially expressed in clinically organ-confined PCa and metastatic PCa tissues; 2) to develop the expression profile of CTAs to predict the aggressiveness of PCa using the CTA-based nCounter Gene Expression Assay; and 3) To test whether the CTAs expression profiling can differentiate the ‘aggressive’ versus ‘indolent’ PCa using blinded samples. In addition to the blinded sample sets, this specific aim will also explore the possibility of using peripheral blood cells that contain circulating tumor cells for assaying the CTAs expression profiling.

2. KEYWORDS

1. Prostate cancer
2. Metastatic prostate cancer
3. Cancer/testis antigen
4. CTA
5. Aggressive
6. Biomarker
7. Gene expression
8. nCounter
9. Nanostring
10. qRT-PCR

3. OVERALL PROJECT SUMMARY

Summary of Tasks in SOW

Tasks	Summarized aims	Time
Major Task 1 Subtasks 1 and 2 Year 1	CTA gene expression analysis by nCounter (Nanostring) and validation by qRT-PCR.	Year 1
Major Task 2 Subtasks 1 and 2 Year 2	1. Confirm CTA expression pattern in 24 tumor extracts of PCa (paired non-tumor and tumor cases). 2. Identify and optimize commercial sources of specific antibodies to perform quantitative IHC using samples organized in training TMAs available in Dr. Veltri's laboratory.	Year 2
Major Task 3 Subtasks 1, 2 and 3	1. Evaluate CTA expression using quantitative (qIHC) for the pre-selected candidates using a TMA containing PCa cases stratified according to Gleason score confirmed by Dr. Jonathan I Epstein. 2. Use uni- and multi-variate logistic regression analysis to create a panel of aberrant expressed CTA that are capable of discriminate and/or predict recurrence for PCa.	Year 3/Year4

Summary of year 1

In the first year of the project (Dr Prakashi Kulkarni was the PI), CTA expression was evaluated using nCounter (Nanostring). A group of 20 localized PCa samples and 20 metastatic cases obtained from Dr. Robert Vessella at University of Washington (Seattle, WA) were used for CTA gene expression analysis. The nCounter results were validated by qRT-PCR, attesting that this multiplex approach may be appropriate for the identification of CTA genes differentially expressed in localized versus metastatic disease.

After completion of the gene expression analysis a change in the project design was requested. In October 2013, as the suggestion of Dr. Kulkarni, it was requested that the PI in charge of the grant be changed from Dr. Prakash Kulkarni to Dr. Robert W Veltri. This change was accepted by the CDMRP in **April, 2014 and W81XWH-12-1-0535 award** was revised. During this process the project was put on hold until a final decision could be made. As soon as the change was accepted, a new postdoctoral fellow (Dr. Luciane T. Kagohara) was hired (July, 2014) and the CTA W81XWH-12-1-0535 project restarted. Due to the delay caused by changes in the project, the Year 2 tasks were delayed and were performed during Year 3.

Summary of year 2 (July/2014 to October/2014)

To identify the best CTA candidates as biomarkers for aggressive prostate cancer (PCa) we performed statistical analysis of the data obtained for CTA gene expression by Nanostring and qRT-PCR of the 20 localized prostate

cancer (LPCa) and 20 metastatic prostate cancer (MPCa) specimens. PRISM software was used to calculate Receiver operator characteristic (ROC) curves to identify a cutoff ratio above the highest control ratio observed for each gene to set specificity at the percentage that maximizes the number of samples correctly classified. Using these cutoff ratios determined by the statistical analysis we then compared the means and verified which CTA pattern of expression were able to discriminate LPCa and MPCa. Combining Nanostring and qRT-PCR ROC curve analysis, our best candidates are: *CEP55, NUF2, PBK, RQCD1, SPAG4, SSX2, TTK* and *PAGE4*.

Summary of year 3 (November 2014 to October 2015)

CTA gene expression analysis in benign adjacent and PCa paired samples was performed by q-RT-PCR. There was no significant difference in the expression pattern of *CEP55, NUF2, PBK, RQCD1* and *TTK* when comparing non-tumor and tumor samples. *PAGE4* was down-regulated in the benign adjacent samples and *SPAG4* and *SSX2* were down-regulated in the tumor areas.
IHC reactions were performed following a protocol well established by Dr. Veltri's research group. Slides were scanned with Aperio Scanning microscope at 20X. IHC reactions for *CEP55, NUF2, PBK, RQCD1, SPAG4, SSX2, TTK* and *PAGE4* were performed using primary antibodies from 2 sources: Sigma-Aldrich and Abcam.
In parallel to CTA expression in prostate tumor and normal tissues, we evaluated the expression profile of *CEP55, NUF2, PBK, RQCD1, SPAG4, SSX2, TTK* and *PAGE4* in PCa cell lines. mRNA and protein expression were determined by qRT-PCR and Western Blot, respectively. For this analysis we selected 6 cell lines: BPH1, DU145, LNCAP, PC3, PC3 Epi and PC3 EMT. Protein expression reflected mRNA levels in the cell lines: positive gene expression resulted in protein detectable levels, while no or low levels of mRNA reflected in negative protein expression.

Year 4 (October 2015 to June 2016) – Final Technical Report

Quantitative immunohistochemistry (qIHC) analysis
During Year 3 of the grant we optimized the IHC reaction for the 8 CTA candidates (*CEP55, NUF2, PBK, RQCD1, SPAG4, SSX2, TTK* and *PAGE4*) using to sources of antibodies: Sigma-Aldrich and Abcam. For the IHC image analysis to quantify the staining signal, we selected the antibody source that resulted in stronger specific staining and weaker unspecific staining (background). In Table 1, we summarized the antibody sources used to measure each CTA protein expression, as well as the dilution factor used for each of them. The IHC protocol, extensively used by Dr Veltri's group was described previously (Year 3 Annual Technical Report). Briefly, deparaffinization of tissue sections was performed in xylene and followed by re-hydration in serial washes in ethanol (100%, 75%, 50% and 25%). Antigen retrieval was performed under heat and adequate pH. After that, steps for endogenous peroxide activity and unspecific protein blocking were performed at room temperature. Incubation with primary antibody was performed overnight at 4^{o}C using pre-optimized dilution. Secondary antibody in a 1:200 dilution was incubated for one hour at room temperature. Staining was performed using DAB substrate solution and counter staining in hematoxylin.
Quantitative IHC was performed using the ImageScope (Aperio) software. The software allows the selection of the areas of interest (cancer or normal areas) and quantifies the intensity and frequency of the positive areas. Intensity is given in number of pixels, while frequency takes into account the proportion between brown versus blue area. Both variables were considered independently during the statistical analysis.
We evaluated expression of the biomarkers and the association with pathology, Gleason score, T stage, PSA for screening, PSA for aggressive disease, biochemical recurrence and tumor margins. For each variable samples were grouped as follows:

- Pathology: cancer or benign;
- Gleason score: 3+3/3+4 or 4+3/higher;

- T stage: T1+T2 or T3+T4;
- PSA screening: ≤4ng/mL or >4ng/mL;
- PSA aggressive disease: ≤10ng/mL or >10ng/mL;
- Biochemical recurrence: no or yes;
- Tumor margins: negative or positive.

To verify if differences in staining intensity and frequency were significantly different we compared medians. We used Wilcoxon non-parametric test for paired samples to compare expression levels from cancer and benign groups, since these samples were collected from the same patient. For the other variables we compared medians by Mann-Whitney non-parametric test, once these variables were related to the same patient but only the cancer areas were considered for analysis. Medians were considered statistically different when $p \leq 0.05$.

We also used Receiver Operator Characteristic (ROC) curve analysis to verify if the expression profiles were capable of separating the two specified groups for each variable and the sensitivity and specificity. A biomarker was considered good to discriminate between the groups when Area Under Curve (AUC)≥0.7.

We then performed multiple logistic regression (MLR) to verify if it was possible to predict the different variables based on expression profile of panels of biomarkers. For this analysis we used backwards-stepwise logistic regression. When AUC≥0.7 the panel of biomarkers left in the model were considered able to predict that specific variable.

Staining intensity and frequency of positive stained cells are significantly higher in cancer cases for all biomarkers when compared to the benign adjacent tissue. AUC for most of the CTA biomarkers was significant or very near to the cut-off, suggesting that they potential biomarkers to discriminate normal from malignant areas (Table 1).

We observed some correlations (Pearson's) between biomarkers expression and the available clinical pathological features:

- CEP55 intensity is significantly higher (p=0.0125) in PCa patients with PSA ≤10ng/mL;
- PAGE4 intensity is significantly higher in PCa GS 4+3/higher (p=0.0327) and PSA ≤10ng/mL (p=0.0151);
- PAGE4 positive cells are more frequent in men with PCa GS 4+3/higher (p=0.0041);
- PBK intensity and frequency are significantly higher in PCa patients with GS 4+3/higher tumors (p=00.35 and p<0.0001, respectively);
- RQCD1 intensity is higher in PCa men with PSA ≤10ng/mL (p=0.0087) and GS 4+3/higher (p=0.0007);
- SPAG4 positive cells frequency is higher in patients with PSA ≤10ng/mL and GS 4+3/higher (p=0.0337 and p=0.0049; respectively);
- SSX2 expression is more frequent in PCa GS 4+3/higher (p=0.0018);
- TTK intensity is higher in PCa patients with PSA ≤10ng/mL (p=0.0358).

SUMMARY: Table 2 and Figure 1 to 16 represent all the described results.

All eight biomarkers selected (CEP55, NUF2, PAGE4, PBK, RQCD1, SPAG4, SSX2 and TTK), besides being highly and frequently expressed on PCa when compared to the benign adjacent tissue, are also correlated with some clinical and pathological features used to determine patients' prognosis. Most of them are associated with advanced GS. However, it is interestingly to verify that most of the proteins are highly expressed in tumors from patients with PSA ≤10ng/mL. According to D'Amico criteria, PSA>10ng/mL is one of the features associated with PCa aggressiveness. Perhaps, our cohort is biased by the fact that it was selected based on the GS, so it would be homogeneous according to this variable and resulted in a cohort that do not reflect a normal distribution when considering PSA levels and other features.

MLR analysis generated some biomarker panels associated with cancer diagnosis and other prognostic variables:

- Cancer areas were discriminated from benign by IHC intensity of CEP55, NUF2, PAGE4, PBK, SPAG4 and TTK (7 biomarkers) with AUC=0.87; specificity=83.1% and sensitivity=70.5% (Figure 17);
- Cancer areas were discriminated from benign by IHC frequency of CEP55, NUF2 and PBK (3 biomarkers) with AUC=0.76; specificity=66.2% and sensitivity=75.4% (Figure 18);

- Cancer areas were discriminated from benign by IHC intensity of all 8 biomarkers and frequency of NUF2, PBK, SSX2 and TTK (AUC=0.96; specificity=89.2% and sensitivity=88.5%) (Figure 19);
- GS 3+3/3+4 were discriminated from GS 4+3/higher by IHC intensity of NUF2, PAGE4, PBK, RQCD1 and TTK and frequency of PAGE4, RQCD1 and TTK (AUC=0.93; specificity=88.0% and sensitivity=88.9%) (Figure 20);
- T stage T1+T2 were discriminated from T3+T4 by IHC intensity of NUF2, PBK, SPAG4 and SSX2 and frequency of CEP55, PBK and SSX2 (AUC=0.78; specificity=66.7% and sensitivity=78.1%) (Figure 21);
- PSA $\leq$10ng/mL was discriminated from >10ng/mL by IHC intensity of NUF2, PBK, SPAG4 and SSX2 and frequency of CEP55, PBK and SSX2 (AUC=0.78; specificity=66.7% and sensitivity=78.1%) (Figure 22);
- Biochemical recurrence was discriminated from absence by IHC intensity of CEP55, PAGE4, PBK and TTK and frequency of CEP55, PAGE4, PBK, SPAG4, SSX2 and TTK (AUC=0.87; specificity=92.5%; sensitivity=57.1%) (Figure 23).

4. KEY RESEARCH ACCOMPLISHMENTS

- Using an automated imaging analysis system (ImageScope, Aperio) we performed quantitative IHC analysis that allowed us to use more statistical tools to determine the potential of our CTA candidates as PCa biomarkers.
- We determined the protein expression profile of 8 CTAs in PCa and normal (benign adjacent) prostate tissue. All biomarker candidates were proven to be over-expressed in cancer when compared to the paired normal tissue (intensity and frequency).
- Higher Gleason score (4+3 and higher) is more frequently among PCa cases with increased levels of PAGE4, PBK, RQCD1, SPAG4 and SSX2. These data suggest that besides being potential screening biomarkers, CTAs can also be useful to predict patients' prognosis.
- There was no association between CTAs expression and the PSA test (cut-off 4ng/mL) used for PCa screening. However, when we considered the cut-off established by D'Amico for aggressive tumors (10ng/mL) we found that CEP55, PAGE4, RQCD1, SPAG4 and TTK were up-regulated in PCa cases with PSA lower or equal to 10ng/mL.
- We identified panels of CTAs which combined protein levels are associated with cancer diagnosis, GS and PSA levels. Using these panels increased specificity and sensitivity than using each biomarker alone. These results indicate that biomarker panels are more accurate to cancer screening and patient prognosis predictions than a single biomarker.

5. REPORTABLE OUTCOMES

All aims were satisfied. CTAs gene expression analysis by Nanostring and qRT-PCR was performed. Differentially expressed CTA genes (*CEP55, NUF2, PAGE4, PBK, RQCD1, SPAG4, SSX2* and *TTK*) have their protein levels evaluated by qIHC in 80 cases with paired PCa and the benign adjacent tissue distributed in TMAs. All eight CTAs presented higher protein levels in tumors than in the non-tumor paired sample. Using multivariate logistic regression we identified panel of biomarkers which expression pattern combined is useful to separate normal from cancer samples and also to predict Gleason score or PSA levels.

6. CONCLUSION

The current study evaluated gene expression profile of CTAs in localized (LPCa) and metastatic prostate cancer (MPCa) samples. Using Nanostring technology and qRT-PCR, we identified 8 CTAs (*CEP55, NUF2, PAGE4, PBK, RQCD1, SPAG4, SSX2, TTK*) that are differentially expressed between the two groups. We then evaluated expression of the same 8 genes in PCa and paired normal tissue (benign adjacent), but for most of these genes there was no significant difference between malignant and benign tissue. We had two hypothesis for these findings: (1) these biomarkers are only differentially expressed in patients with MPCa or (2) other regulatory mechanisms altered during cancer progression would result in increased translation of these genes, reflecting in increased protein expression without mRNA up-regulation.

To verify if there are differences in protein levels of the 8 CTA candidates, we performed qIHC in 80 paired cases (PCa and benign adjacent tissue) distributed in 2 TMA-681-682 (Veltri Lab product): all blocks were selected and stratified according to the most current Gleason score standards by Dr. Jonathan I. Epstein. It would also give us the chance to verify the expression of these proteins across different disease stages.

We found that all the CTAs selected are highly expressed in cancer when compared to the normal tissue from the same patient. Also, the expression of some of them (PAGE4, PBK, RQCD1, SPAG4, SSX2) is increased in PCa GS 4+3 and higher, suggesting that these biomarkers increase with disease progression.

Here, we identified genes that are ideal tumor biomarkers. CTAs are expressed only by cancer cells, except by normal testis that is an immune-privileged organ. Also, they are capable of inducing an immune response and though are good targets for the development of immunotherapy treatments. The proteins encoded by the CTAs *CEP55, NUF2, PAGE4, PBK, RQCD1, SPAG4, SSX2* and *TTK* are highly expressed in PCa. Also, these genes have differential expression between LPCa and MPCa. These data together suggest that these biomarkers could be used as a screening test and also as a follow-up tool to determine which patients will progress with MPCa.

7. PUBLICATIONS, ABSTRACTS AND PRESENTATIONS

- 2016 AACR Annual Meeting
  Kagohara LT, Mooney S, Chen Y, Orban J, Kulkarni P, Veltri RW. Phosphorylation of the intrinsically disordered cancer/testis antigen PAGE4 by HIPK1 and CLK2 in prostate cancer.

8. INVENTIONS, PATENTS AND LICENSES

Nothing to report.

9. OTHER ACHIEVEMENTS

In a previous study conducted at The Brady Urological Institute at Johns Hopkins Medical Institutions, PAGE4 was shown to be phosphorylated by HIPK1, resulting in increased transactivation of c-Jun (Mooney et al. 2014). Another kinase that phosphorylates PAGE4 in a different residue is CLK2 (unpublished data). To verify the expression of these two kinases and the correlation with PAGE4 expression, we performed qIHC using the same TMA cases. As observed for PAGE4, expression of CLK2 and HIPK1 were stronger and more frequent in PCa cases, when compared to normal adjacent prostate tissue (Figure 24). These results suggest that in PCa increased levels of PAGE4 are associated with higher levels of CLK2 and HIPK1. PCa cases with Gleason Score 4+3 or higher presented higher levels of HIPK1 than cases with lower scores (Figure 25), suggesting that phosphorylation of PAGE4 by this kinase result in the development of more aggressive tumors. Statistical analysis to verify the association with other clinical and pathological features are underway and also further studies using NMR to determine how the kinases interact with PAGE4 are in the final steps and will be concluded for publication.

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Scanlan MJ, Simpson AJ, Old LJ. The cancer/testis genes: review, standardization, and commentary. Cancer Immun. 2004 Jan 23;4:1.

Mooney SM, Qiu R, Kim JJ, Sacho EJ, Rajagopalan K, Johng D, Shiraishi T, Kulkarni P, Weninger KR. Cancer/testis antigen PAGE4, a regulator of c-Jun transactivation, is phosphorylated by homeodomain-

interacting protein kinase 1, a component of the stress-response pathway. Biochemistry. 2014 Mar 18;53(10):1670-9.

11. APPENDICES

Nothing to report.

12. SUPPORTING DATA

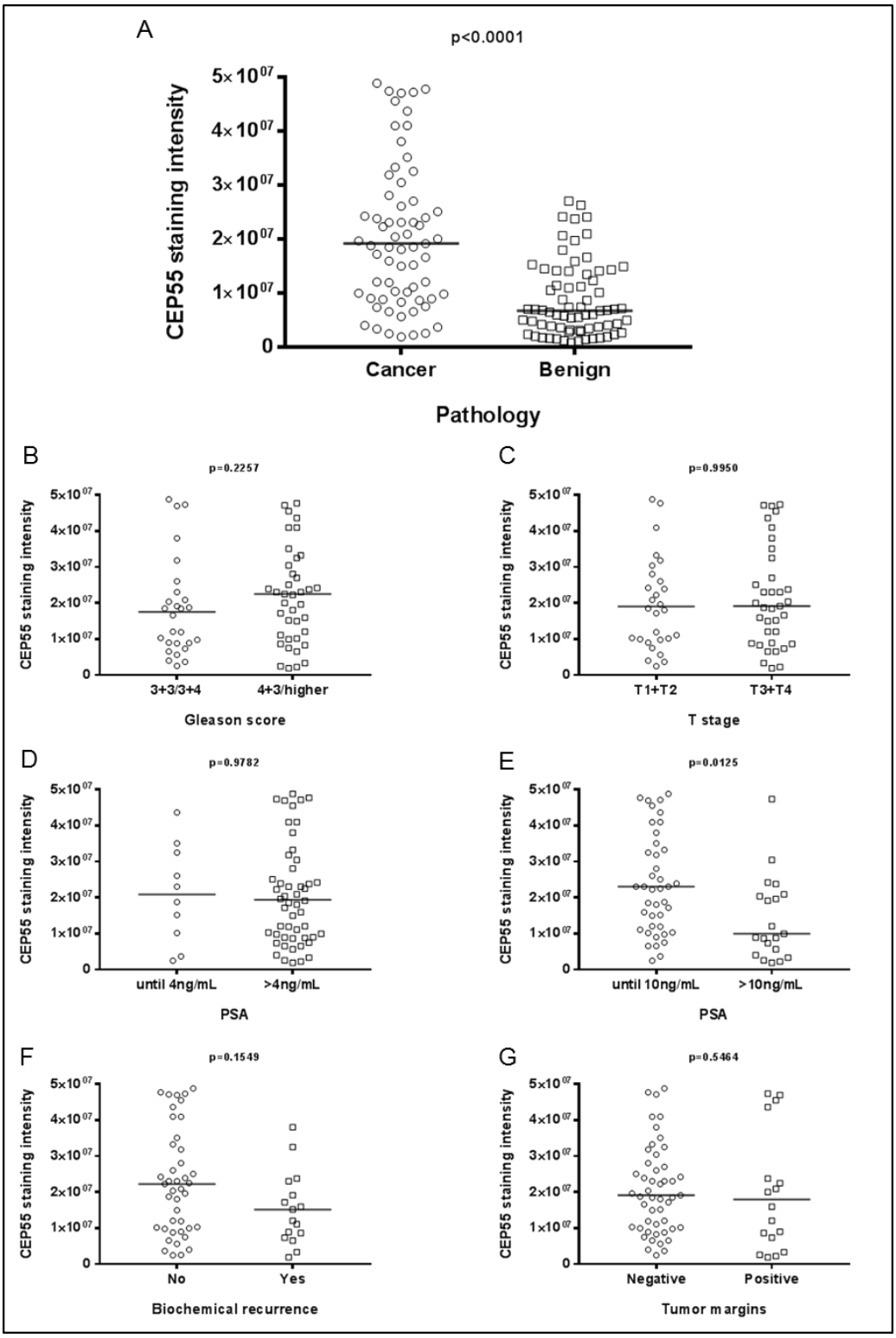


FIGURE 1 – CEP55 immunohistochemistry staining intensity across clinical and pathological variables. Scatter plots represent IHC staining for the cancer samples and/or benign adjacent tissue. CEP55 intensity is significantly higher ($p<0.0001$; Wilcoxon non-parametric test) in the cancer area when compared to the same patient benign prostate tissue (A). When the cancer samples were grouped according to Gleason score (B), T stage (C), PSA for screening (D), PSA for aggressiveness prognosis (E), biochemical recurrence (F) and tumor surgical margins (G) we found that CEP55 is significantly higher in PCa patients with PSA levels <10ng/mL ($p=0.0125$; Mann-Whitney non-parametric test).

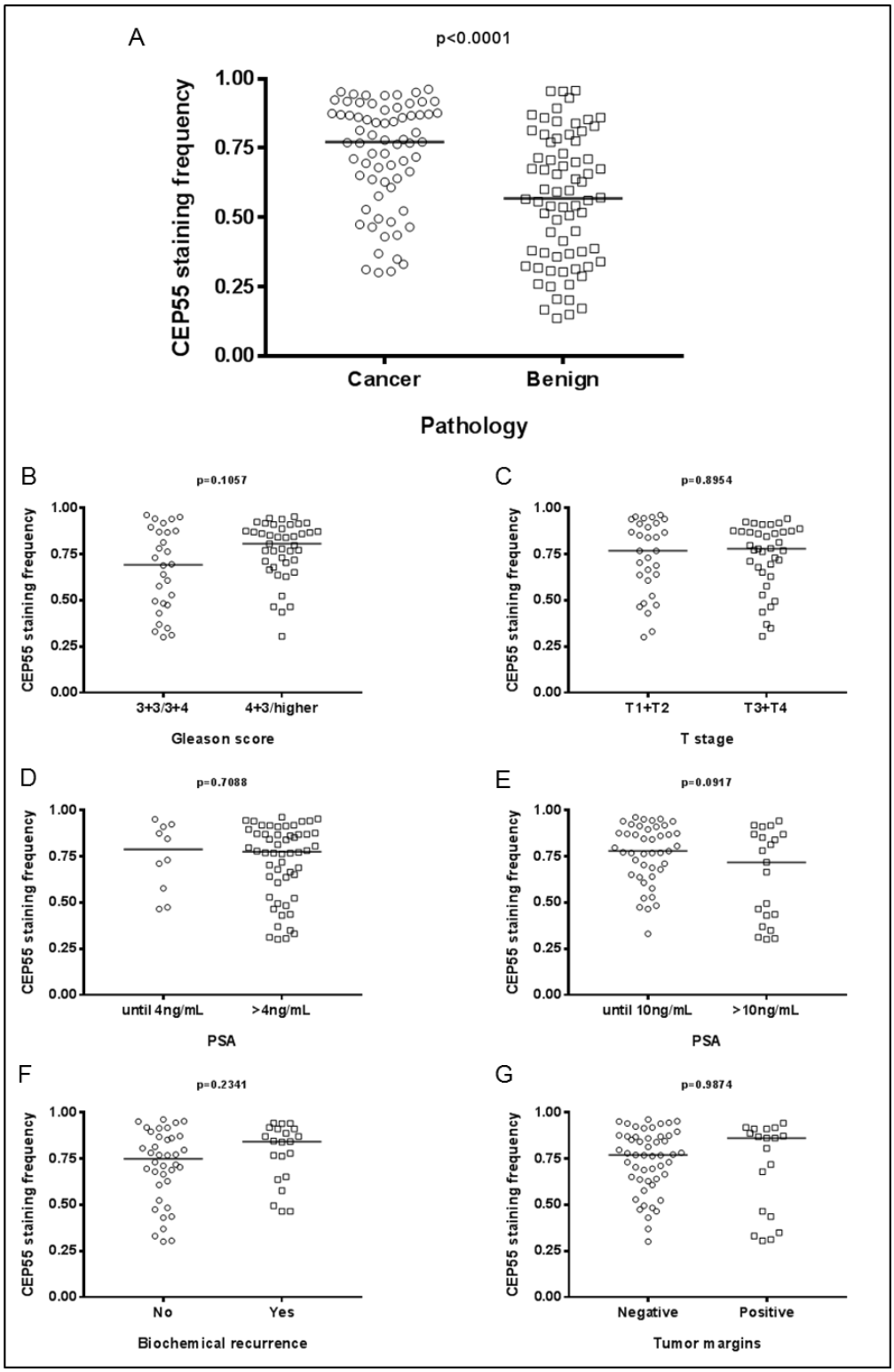


FIGURE 2 – CEP55 immunohistochemistry staining frequency across clinical and pathological variables. Scatter plots represent IHC staining for the cancer samples and/or benign adjacent tissue. CEP55 positive cells are significantly more frequent ($p<0.0001$; Wilcoxon non-parametric test) in the cancer area when compared to the same patient benign prostate tissue (A). When the cancer samples were grouped according to Gleason score (B), T stage (C), PSA for screening (D), PSA for aggressiveness prognosis (E), biochemical recurrence (F) and tumor surgical margins (G) we found no difference in CEP55 expression (Mann-Whitney non-parametric test).

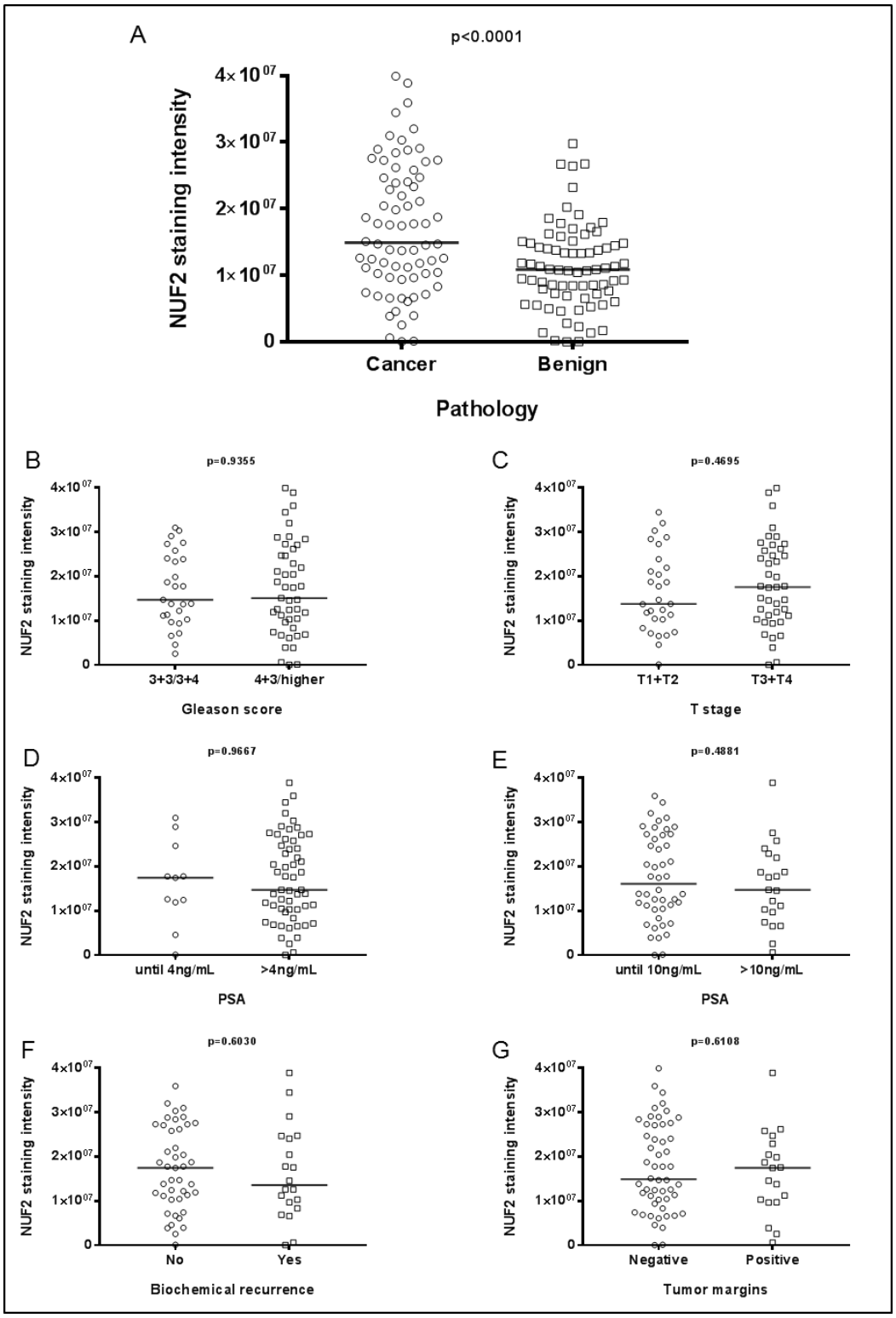


FIGURE 3 – NUF2 immunohistochemistry staining intensity across clinical and pathological variables. Scatter plots represent IHC staining for the cancer samples and/or benign adjacent tissue. NUF2 intensity is significantly higher ($p<0.0001$; Wilcoxon non-parametric test) in the cancer area when compared to the same patient benign prostate tissue (A). When the cancer samples were grouped according to Gleason score (B), T stage (C), PSA for screening (D), PSA for aggressiveness prognosis (E), biochemical recurrence (F) and tumor surgical margins (G) we found no difference in NUF2 expression (Mann-Whitney non-parametric test).

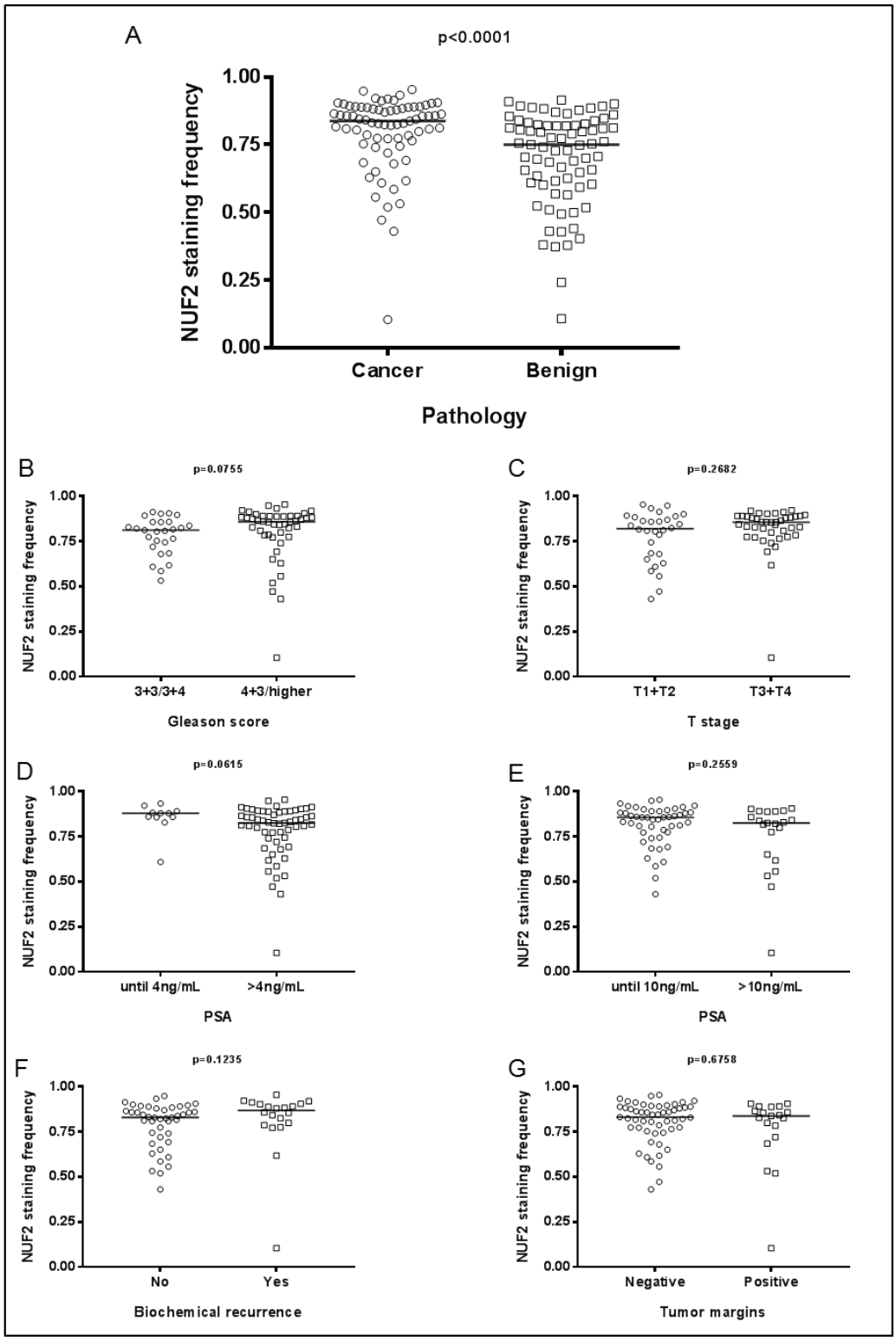


FIGURE 4 – NUF2 immunohistochemistry staining frequency across clinical and pathological variables. Scatter plots represent IHC staining for the cancer samples and/or benign adjacent tissue. NUF2 positive cells are significantly more frequent ($p<0.0001$; Wilcoxon non-parametric test) in the cancer area when compared to the same patient benign prostate tissue (A). When the cancer samples were grouped according to Gleason score (B), T stage (C), PSA for screening (D), PSA for aggressiveness prognosis (E), biochemical recurrence (F) and tumor surgical margins (G) we found no difference in NUF2 expression (Mann-Whitney non-parametric test).

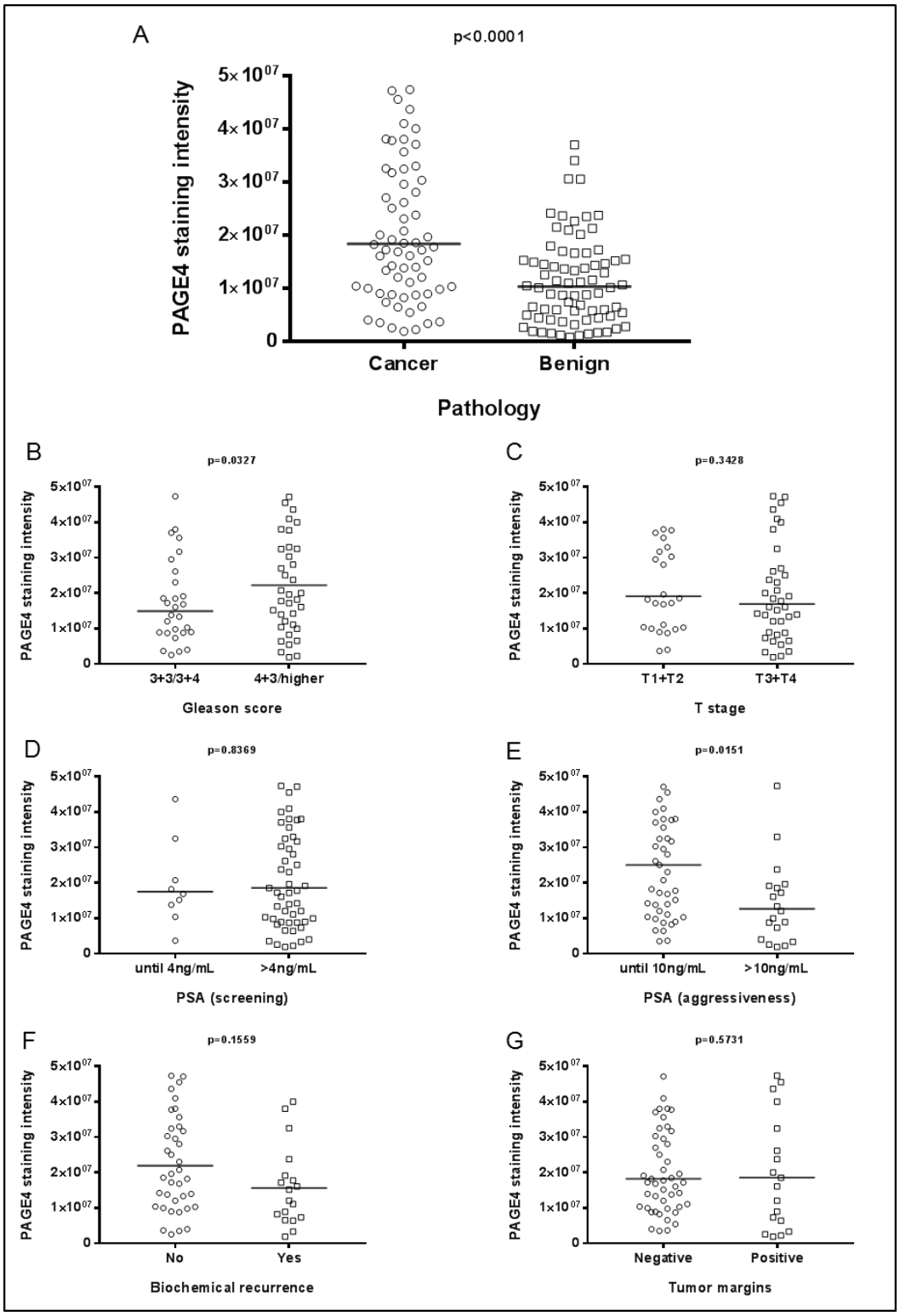


FIGURE 5 – PAGE4 immunohistochemistry staining intensity across clinical and pathological variables. Scatter plots represent IHC staining for the cancer samples and/or benign adjacent tissue. PAGE4 intensity is significantly higher ($p<0.0001$; Wilcoxon non-parametric test) in the cancer area when compared to the same patient benign prostate tissue (A). When the cancer samples were grouped according to Gleason score (B), T stage (C), PSA for screening (D), PSA for aggressiveness prognosis (E), biochemical recurrence (F) and tumor surgical margins (G) we found that PAGE4 is significantly higher in PCa patients with Gleason score 4+3 or higher ($p=0.0327$; Mann-Whitney non-parametric test).

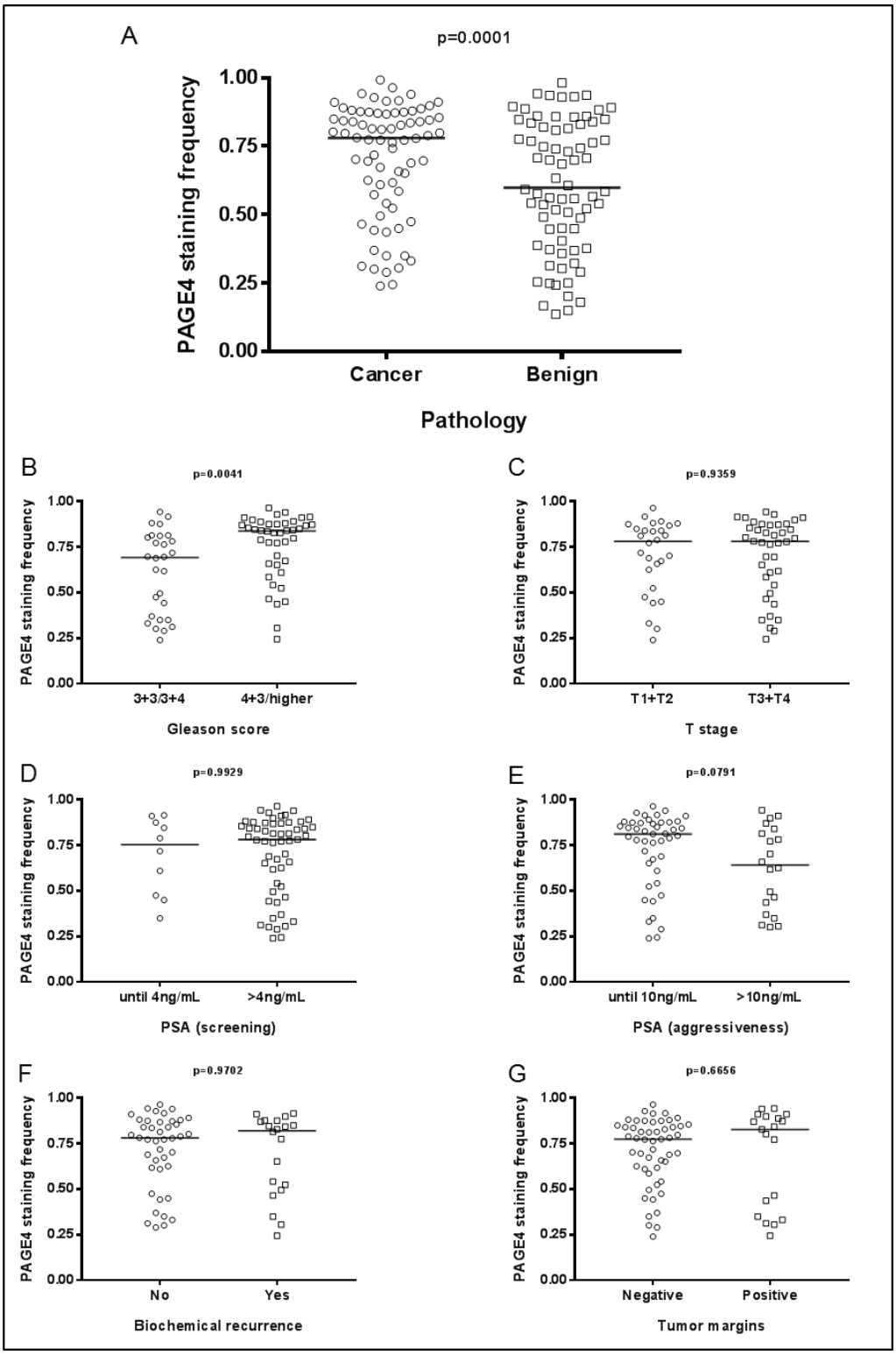


FIGURE 6 – PAGE4 immunohistochemistry staining frequency across clinical and pathological variables. Scatter plots represent IHC staining for the cancer samples and/or benign adjacent tissue. PAGE4 positive cells are significantly more frequent (p=0.0001; Wilcoxon non-parametric test) in the cancer area when compared to the same patient benign prostate tissue (A). When the cancer samples were grouped according to Gleason score (B), T stage (C), PSA for screening (D), PSA for aggressiveness prognosis (E), biochemical recurrence (F) and tumor surgical margins (G) we found that PAGE4 expression is more frequent in PCa cases Gleason score 4+3 or higher (p=0.0041; Mann-Whitney non-parametric test).

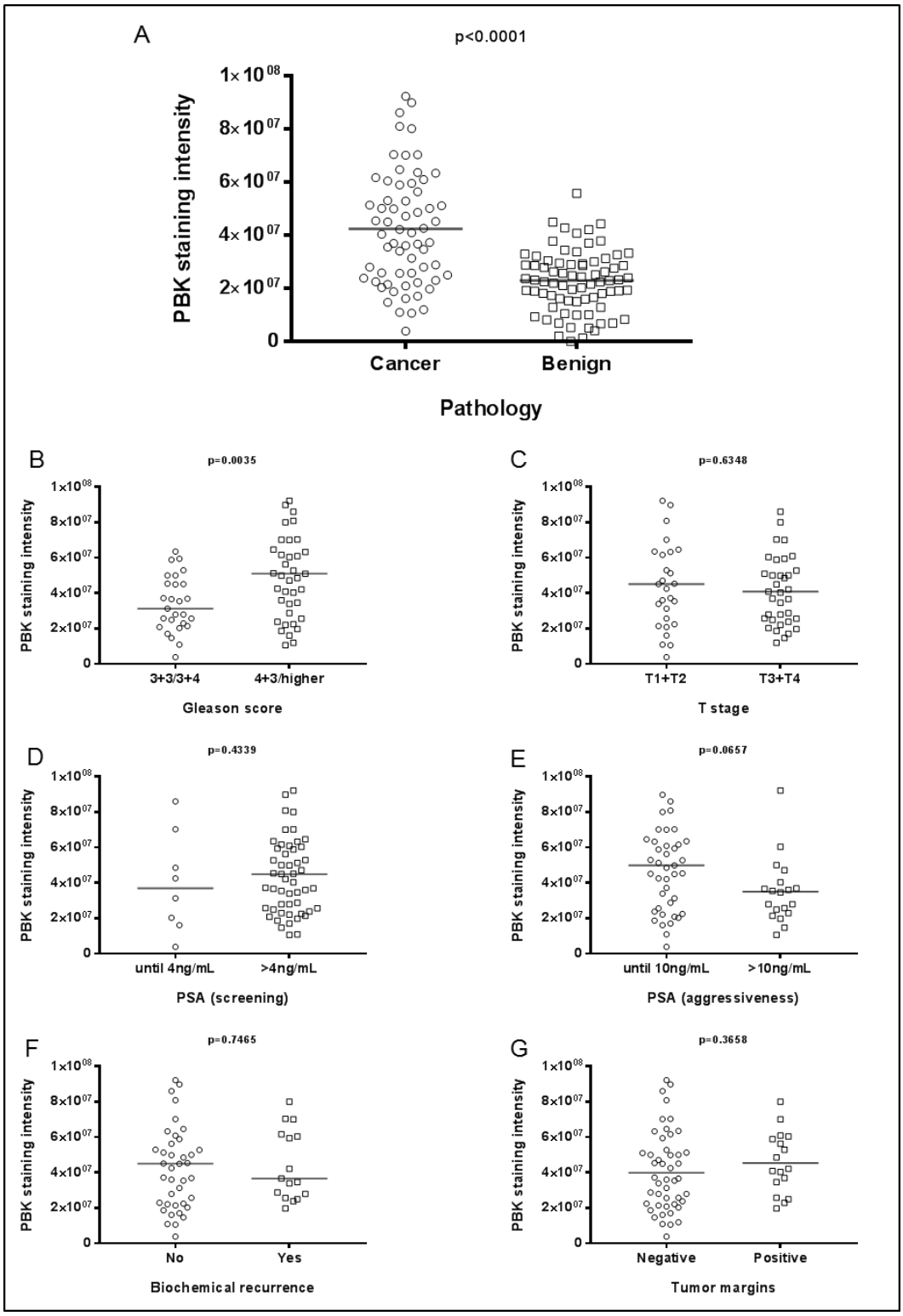


FIGURE 7 – PBK immunohistochemistry staining intensity across clinical and pathological variables. Scatter plots represent IHC staining for the cancer samples and/or benign adjacent tissue. PBK intensity is significantly higher ($p<0.0001$; Wilcoxon non-parametric test) in the cancer area when compared to the same patient benign prostate tissue (A). When the cancer samples were grouped according to Gleason score (B), T stage (C), PSA for screening (D), PSA for aggressiveness prognosis (E), biochemical recurrence (F) and tumor surgical margins (G) we found that PBK is significantly higher in PCa patients with Gleason score 4+3 or higher ($p=0.0035$; Mann-Whitney non-parametric test).

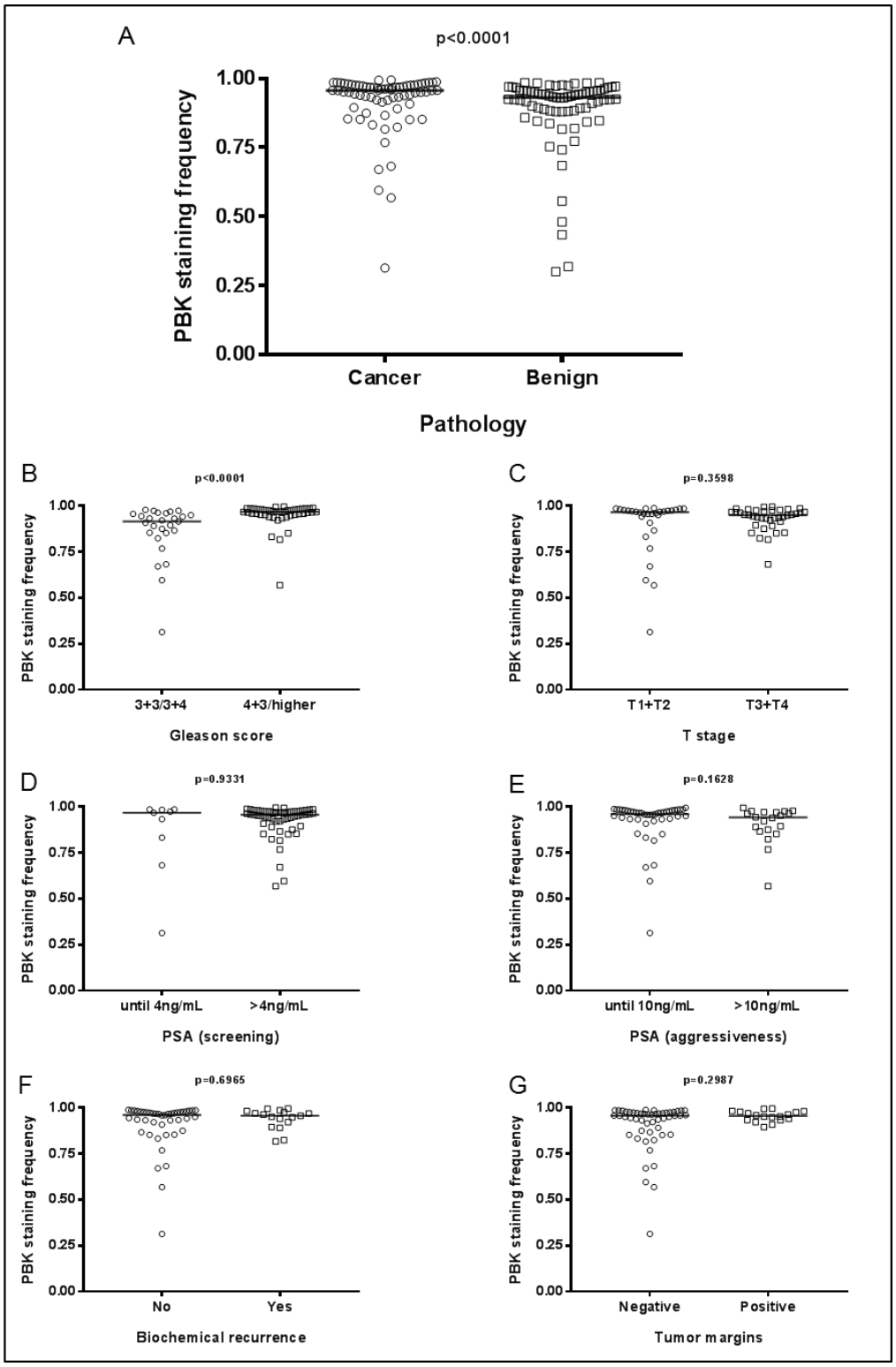


FIGURE 8 – PBK immunohistochemistry staining frequency across clinical and pathological variables. Scatter plots represent IHC staining for the cancer samples and/or benign adjacent tissue. PBK positive cells are significantly more frequent ($p<0.0001$; Wilcoxon non-parametric test) in the cancer area when compared to the same patient benign prostate tissue (A). When the cancer samples were grouped according to Gleason score (B), T stage (C), PSA for screening (D), PSA for aggressiveness prognosis (E), biochemical recurrence (F) and tumor surgical margins (G) we found that PBK expression is more frequent in PCa cases Gleason score 4+3 or higher ($p<0.0001$; Mann-Whitney non-parametric test).

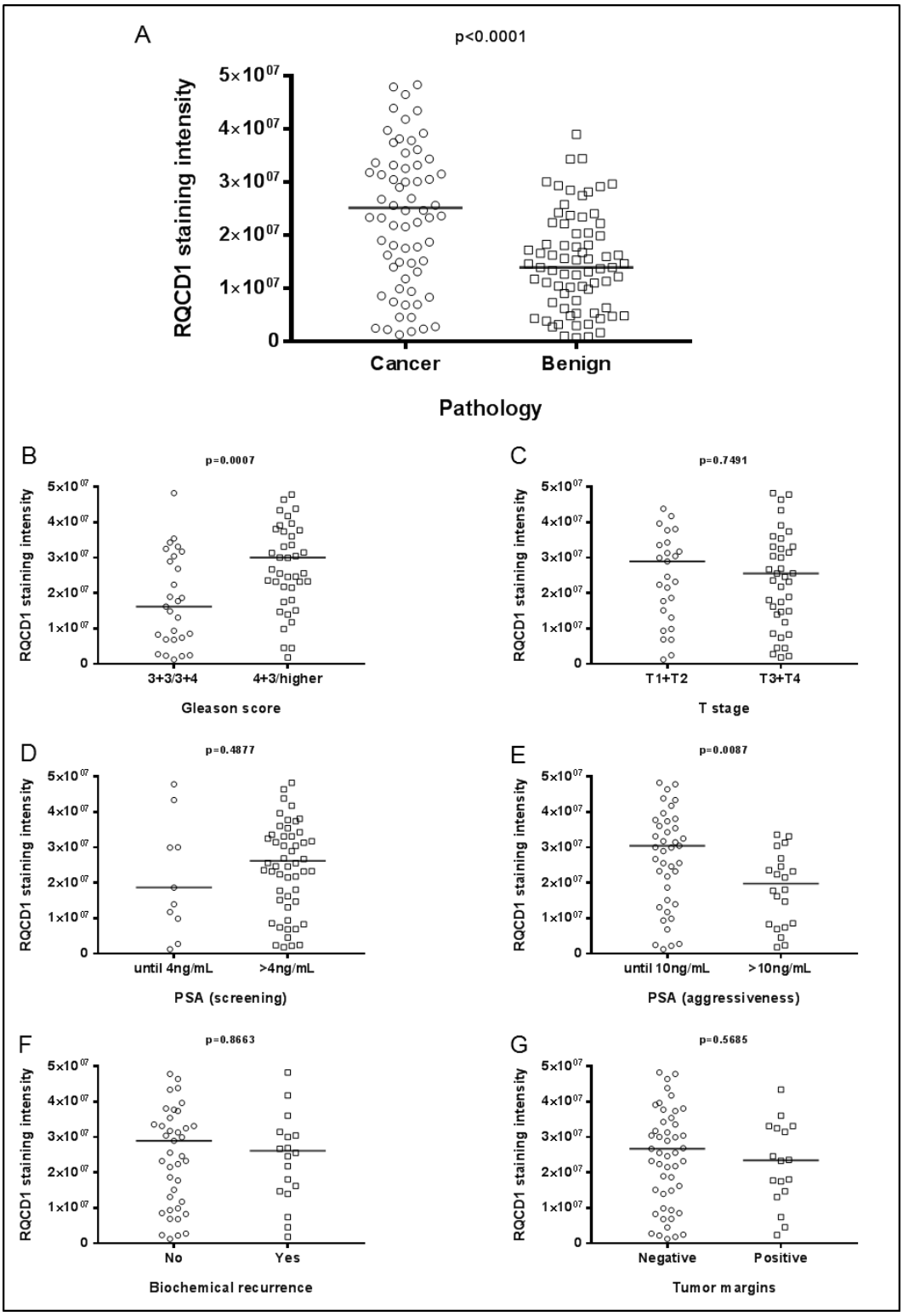


FIGURE 9 – RQCD1 immunohistochemistry staining intensity across clinical and pathological variables. Scatter plots represent IHC staining for the cancer samples and/or benign adjacent tissue. RQCD1 intensity is significantly higher ($p<0.0001$; Wilcoxon non-parametric test) in the cancer area when compared to the same patient benign prostate tissue (A). When the cancer samples were grouped according to Gleason score (B), T stage (C), PSA for screening (D), PSA for aggressiveness prognosis (E), biochemical recurrence (F) and tumor surgical margins (G) we found that RQCD1 expression is significantly higher in PCa patients with PSA levels $\leq$10ng/mL and in those cases with Gleason score 4+3 or higher ($p=0.0087$ and $p=0.0007$ respectively; Mann-Whitney non-parametric test).

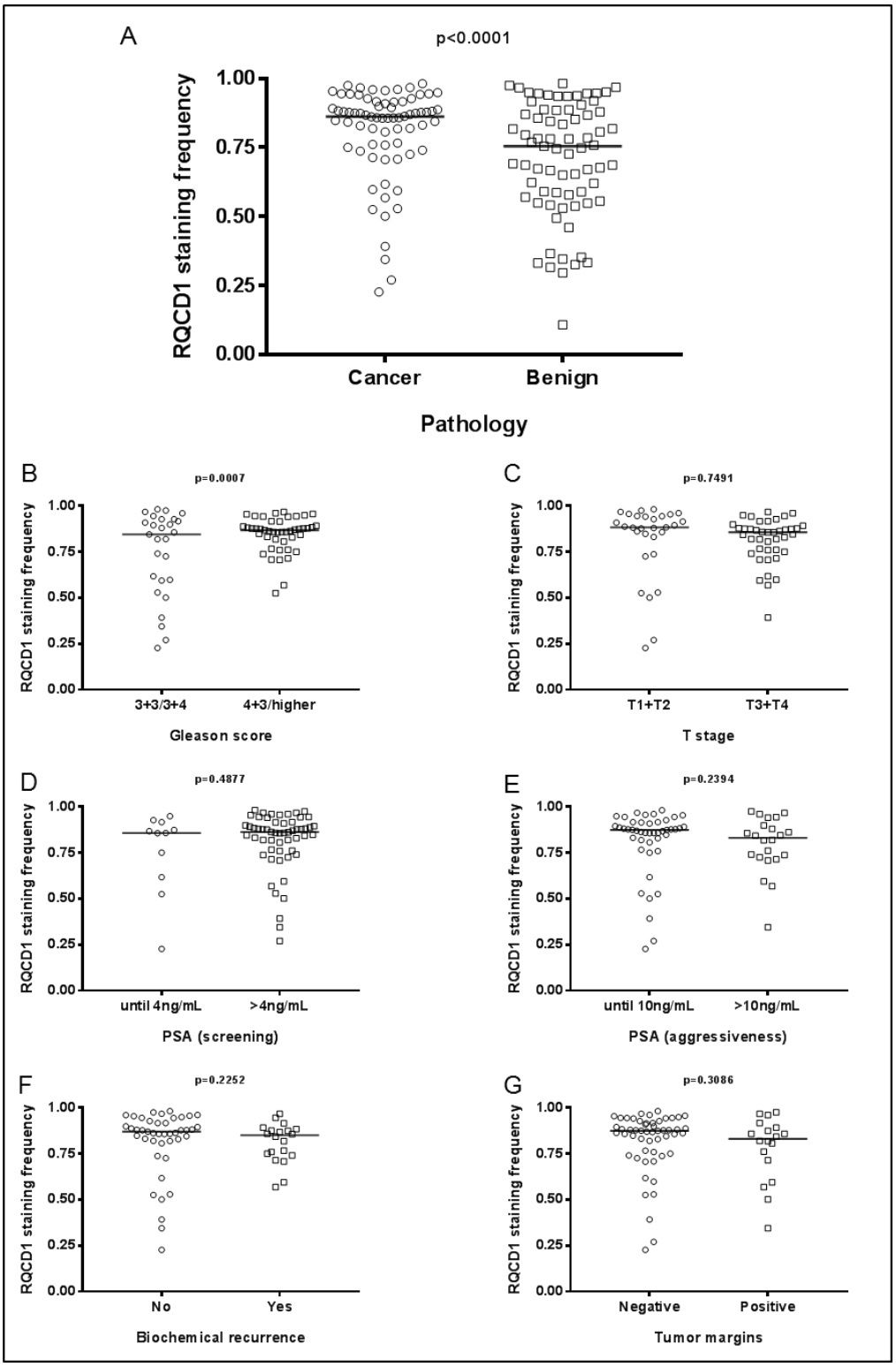


FIGURE 10 – RQCD1 immunohistochemistry staining frequency across clinical and pathological variables. Scatter plots represent IHC staining for the cancer samples and/or benign adjacent tissue. RQCD1 positive cells are significantly more frequent ($p<0.0001$; Wilcoxon non-parametric test) in the cancer area when compared to the same patient benign prostate tissue (A). When the cancer samples were grouped according to Gleason score (B), T stage (C), PSA for screening (D), PSA for aggressiveness prognosis (E), biochemical recurrence (F) and tumor surgical margins (G) we found no difference in RQCD1 expression (Mann-Whitney non-parametric test).

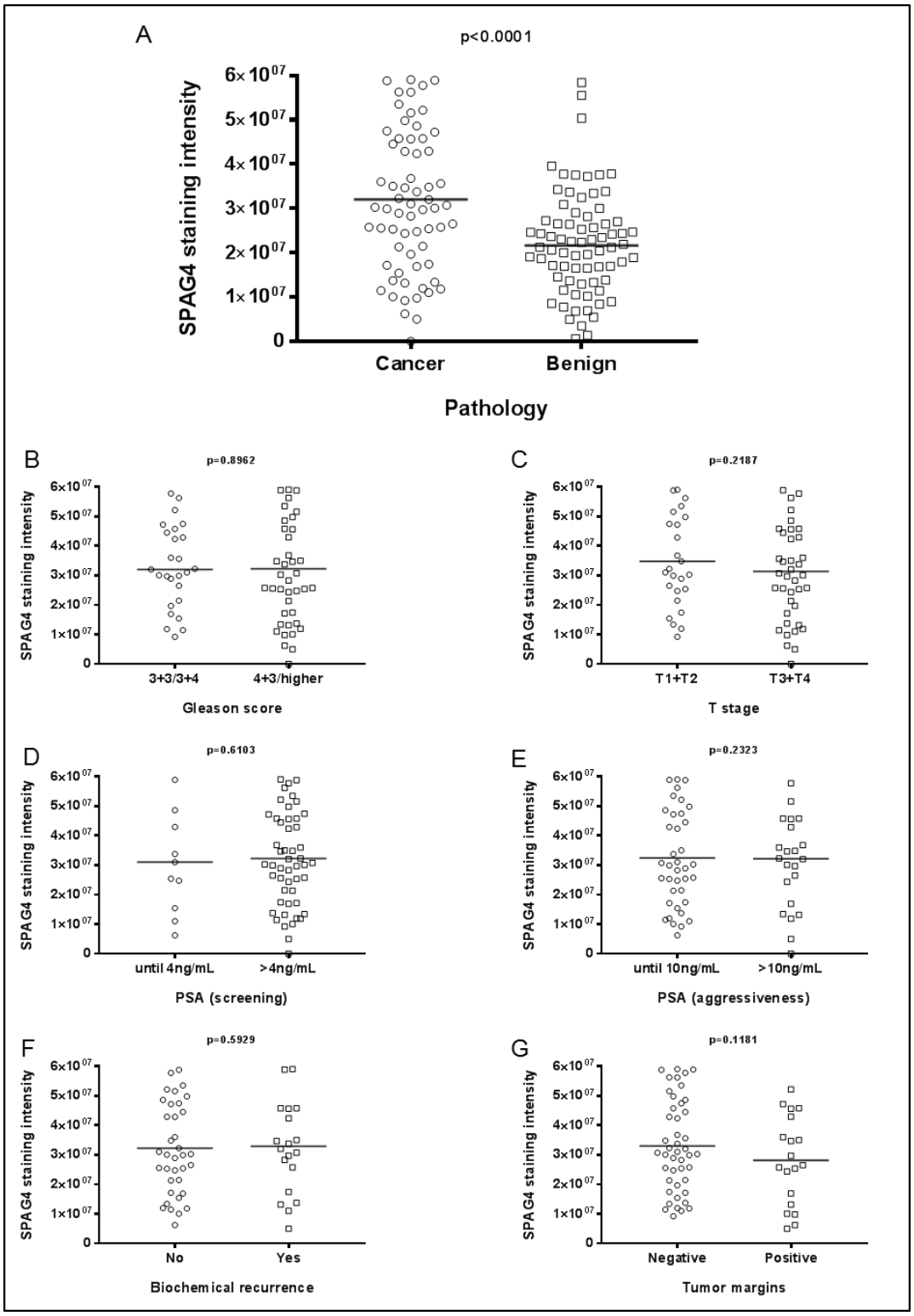


FIGURE 11 – SPAG4 immunohistochemistry staining intensity across clinical and pathological variables. Scatter plots represent IHC staining for the cancer samples and/or benign adjacent tissue. SPAG4 intensity is significantly higher ($p<0.0001$; Wilcoxon non-parametric test) in the cancer area when compared to the same patient benign prostate tissue (A). When the cancer samples were grouped according to Gleason score (B), T stage (C), PSA for screening (D), PSA for aggressiveness prognosis (E), biochemical recurrence (F) and tumor surgical margins (G) we found no difference in SPAG4 expression (Mann-Whitney non-parametric test).

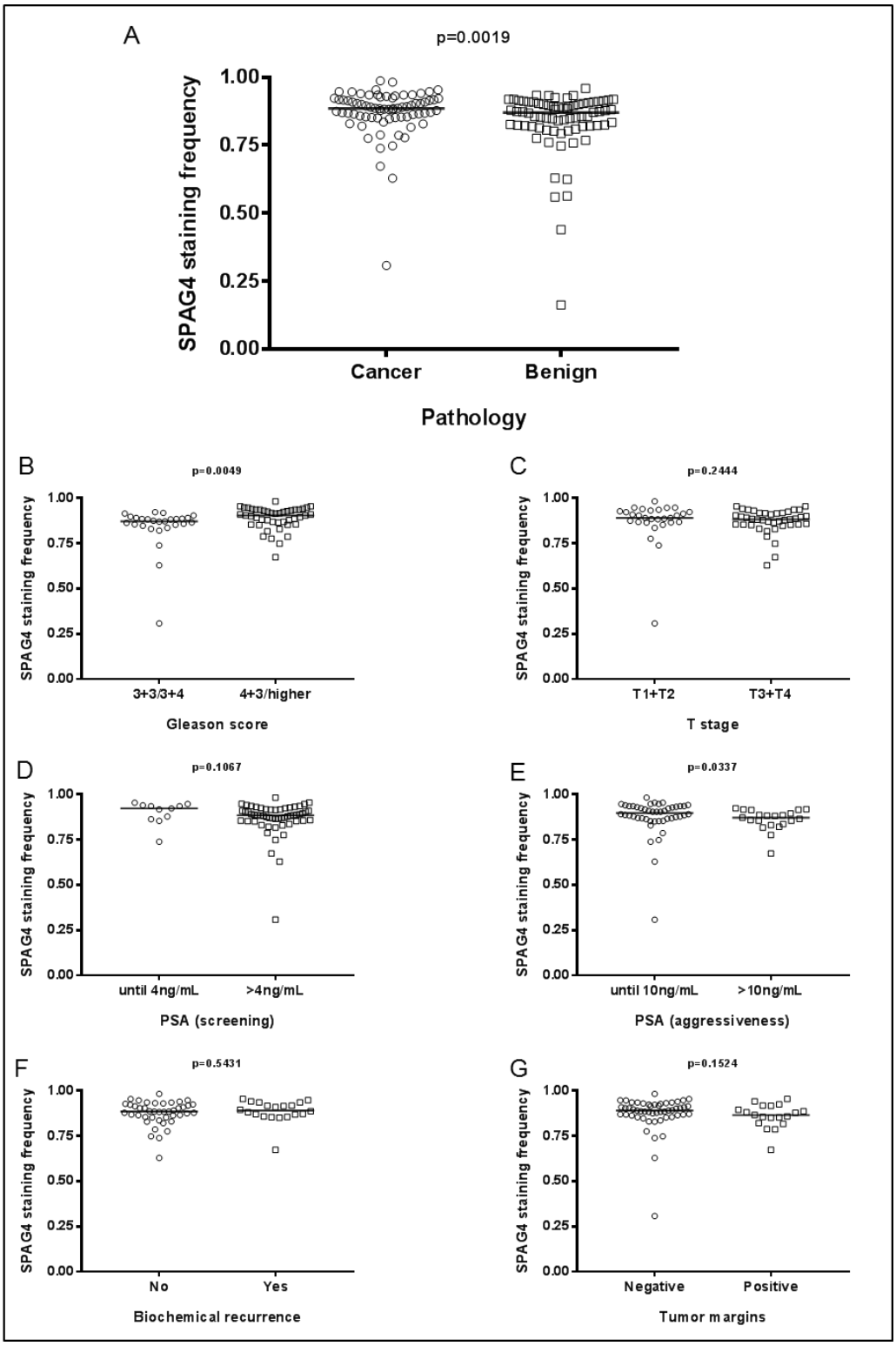


FIGURE 12 – SPAG4 immunohistochemistry staining frequency across clinical and pathological variables. Scatter plots represent IHC staining for the cancer samples and/or benign adjacent tissue. SPAG4 positive cells are significantly more frequent (p=0.0019; Wilcoxon non-parametric test) in the cancer area when compared to the same patient benign prostate tissue (A). When the cancer samples were grouped according to Gleason score (B), T stage (C), PSA for screening (D), PSA for aggressiveness prognosis (E), biochemical recurrence (F) and tumor surgical margins (G) we found that SPAG4 expression is significantly more frequent in PCa patients with PSA levels $\leq$10ng/mL and in those cases with Gleason score 4+3 or higher (p=0.0337 and p=0.0049 respectively; Mann-Whitney non-parametric test).

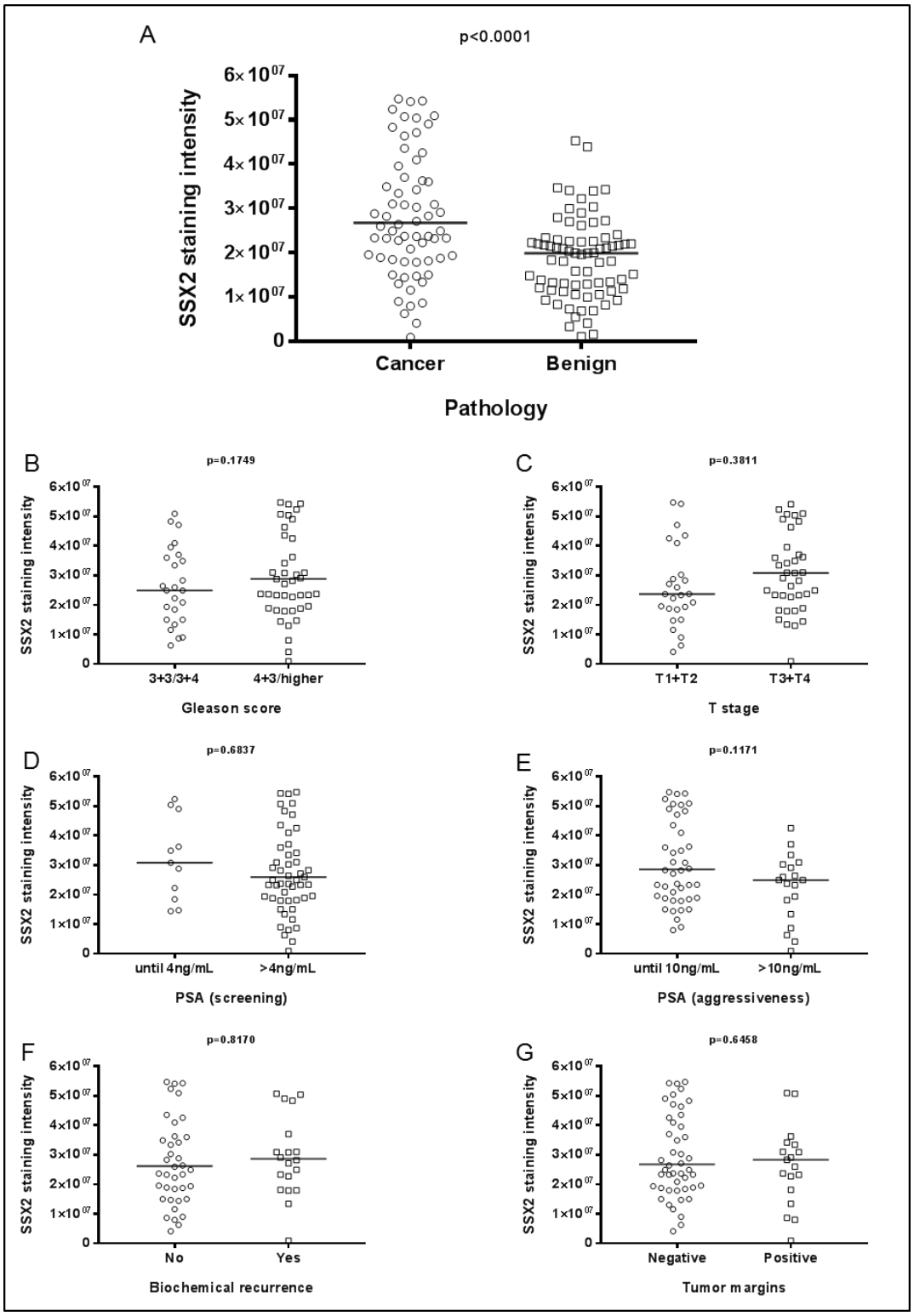


FIGURE 13 – SSX2 immunohistochemistry staining intensity across clinical and pathological variables. Scatter plots represent IHC staining for the cancer samples and/or benign adjacent tissue. SSX2 intensity is significantly higher ($p<0.0001$; Wilcoxon non-parametric test) in the cancer area when compared to the same patient benign prostate tissue (A). When the cancer samples were grouped according to Gleason score (B), T stage (C), PSA for screening (D), PSA for aggressiveness prognosis (E), biochemical recurrence (F) and tumor surgical margins (G) we found no difference in SSX2 expression (Mann-Whitney non-parametric test).

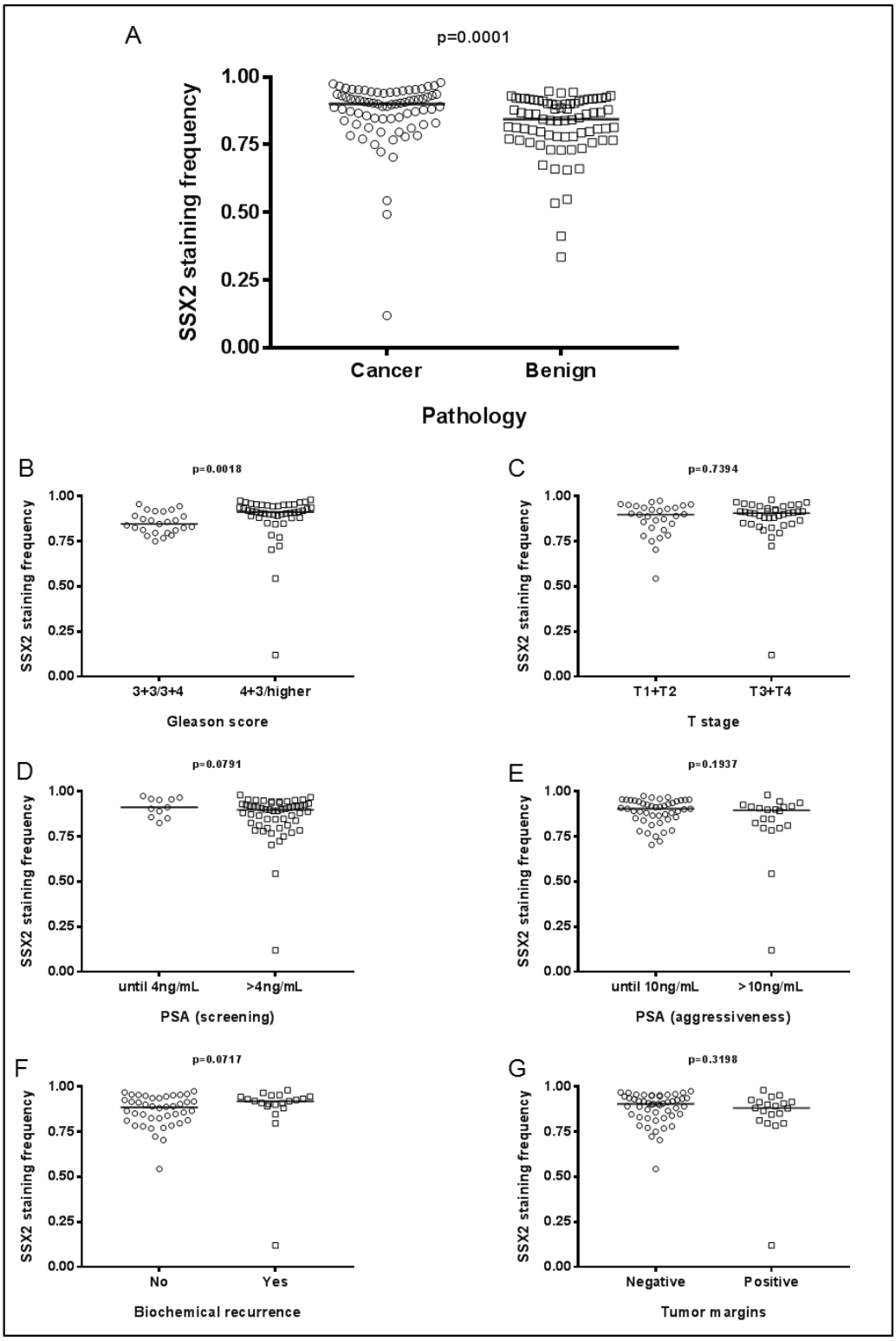


FIGURE 14 – SSX2 immunohistochemistry staining frequency across clinical and pathological variables. Scatter plots represent IHC staining for the cancer samples and/or benign adjacent tissue. SSX2 positive cells are significantly more frequent (p=0.0001; Wilcoxon non-parametric test) in the cancer area when compared to the same patient benign prostate tissue (A). When the cancer samples were grouped according to Gleason score (B), T stage (C), PSA for screening (D), PSA for aggressiveness prognosis (E), biochemical recurrence (F) and tumor surgical margins (G) we found that SSX2 expression is significantly more frequent in PCa patients with Gleason score 4+3 or higher (p=0.0018; Mann-Whitney non-parametric test).

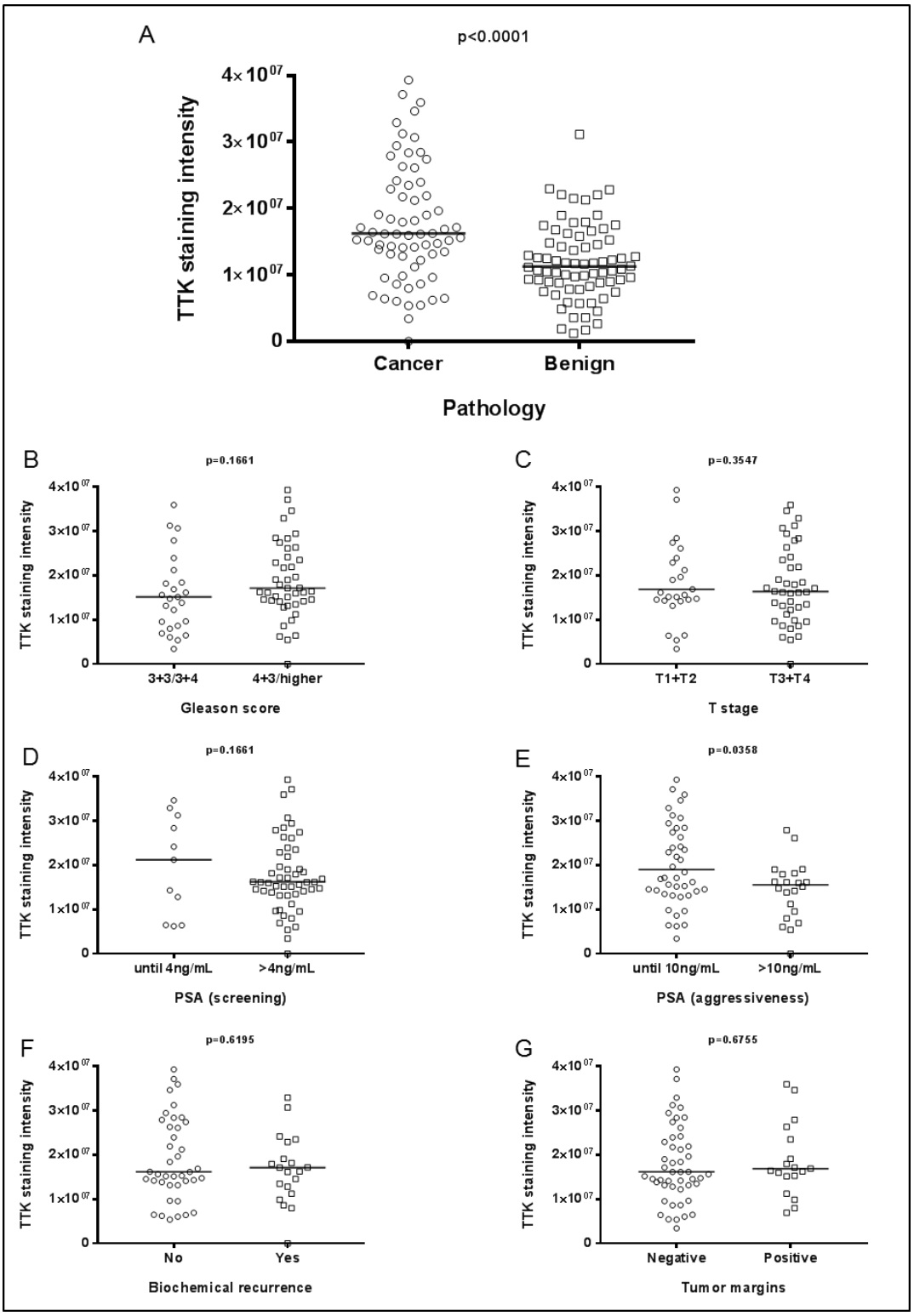


FIGURE 15 – TTK immunohistochemistry staining intensity across clinical and pathological variables. Scatter plots represent IHC staining for the cancer samples and/or benign adjacent tissue. TTK intensity is significantly higher ($p<0.0001$; Wilcoxon non-parametric test) in the cancer area when compared to the same patient benign prostate tissue (A). When the cancer samples were grouped according to Gleason score (B), T stage (C), PSA for screening (D), PSA for aggressiveness prognosis (E), biochemical recurrence (F) and tumor surgical margins (G) we found that TTK expression is significantly higher in PCa patients with PSA levels $\leq$10ng/mL ($p=0.0358$; Mann-Whitney non-parametric test).

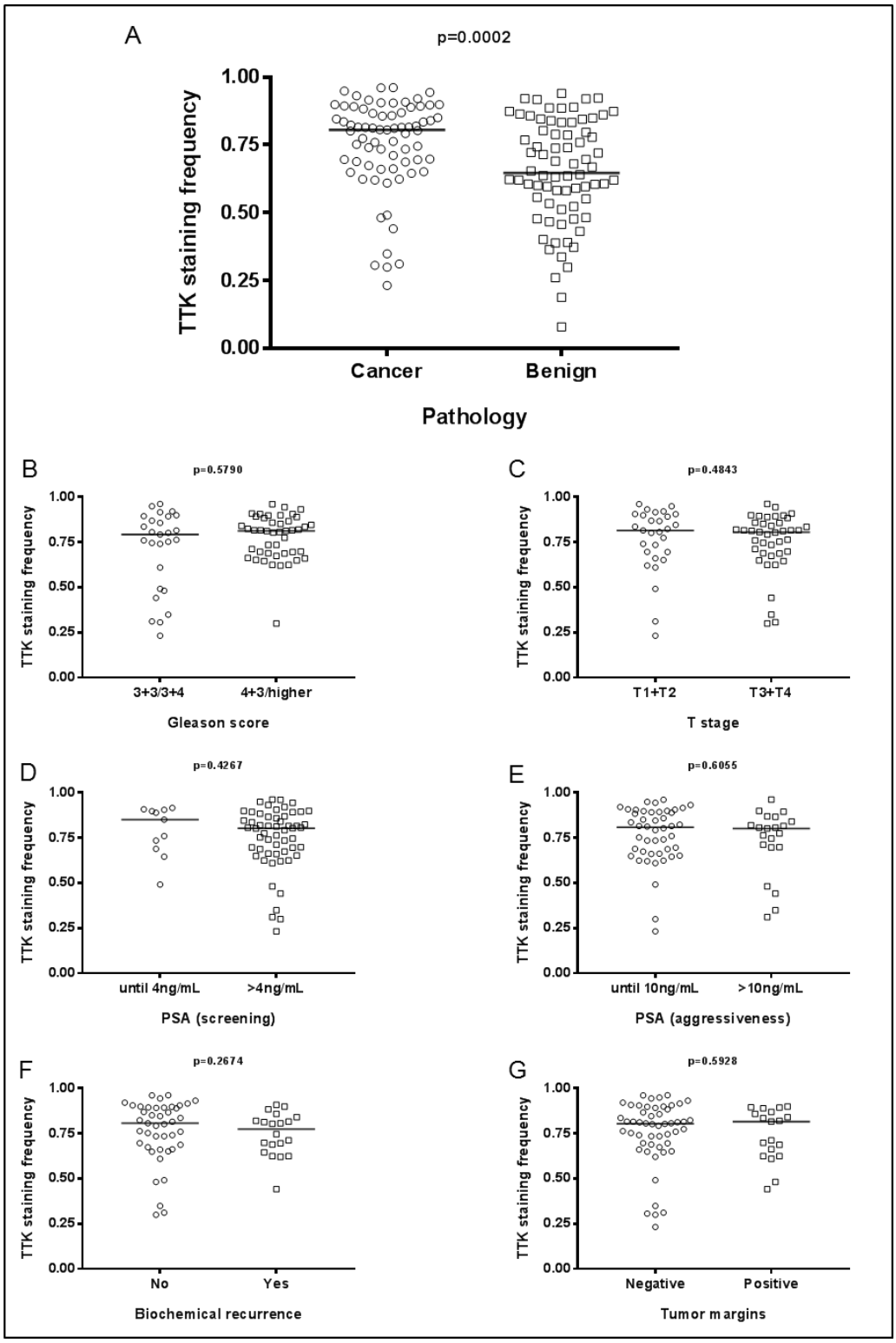
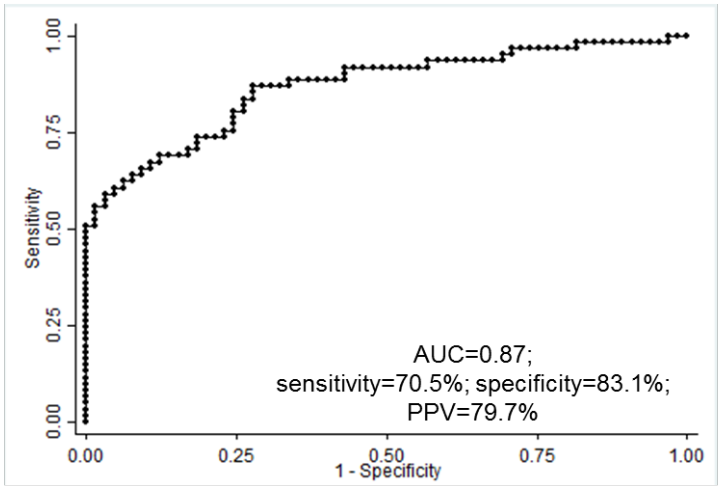
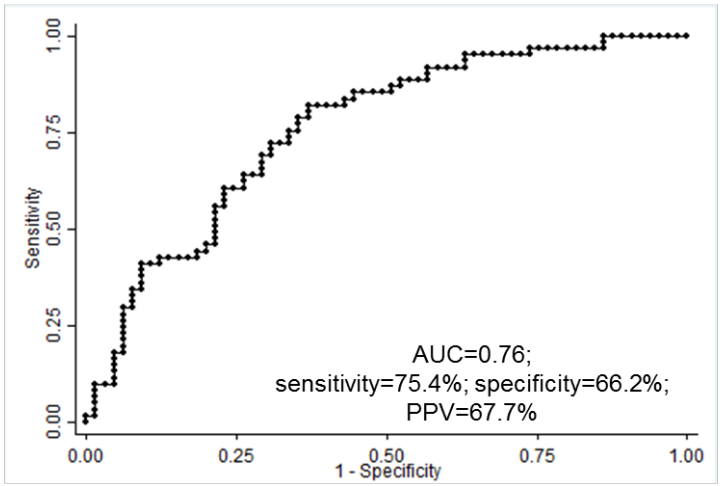


FIGURE 16 – TTK immunohistochemistry staining frequency across clinical and pathological variables. Scatter plots represent IHC staining for the cancer samples and/or benign adjacent tissue. TTK positive cells are significantly more frequent (p=0.0002; Wilcoxon non-parametric test) in the cancer area when compared to the same patient benign prostate tissue (A). When the cancer samples were grouped according to Gleason score (B), T stage (C), PSA for screening (D), PSA for aggressiveness prognosis (E), biochemical recurrence (F) and tumor surgical margins (G) we found no difference in TTK expression (Mann-Whitney non-parametric test).



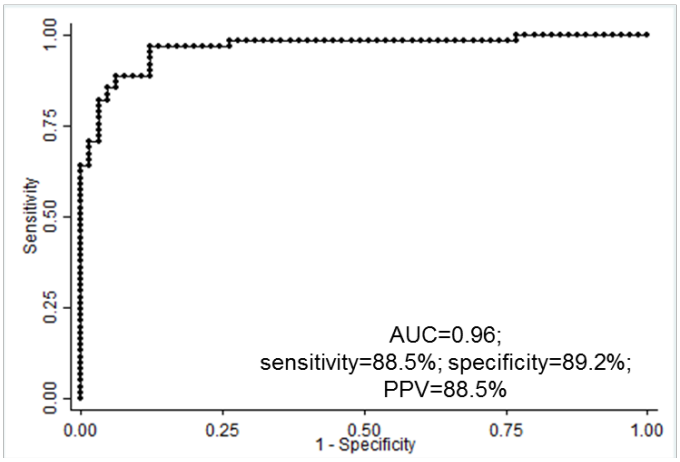
Variables: IHC intensity vs. pathology (cancer/benign)
Panel: CEP55, NUF2, PAGE4, PBK, RQCD1, SPAG4 and TTK

FIGURE 17 – ROC curve from multivariate logistic regression (MLR) for IHC intensity vs. pathology (cancer/benign). MLR was performed using the stepwise backwards logistic regression for pr=0.20. IHC intensity for seven out of eight biomarkers (CEP55, NUF2, PAGE4, PBK, RQCD1, SPAG4 and TTK) resulted in a test with high sensitivity and specificity to discriminate cancer from benign tissue.



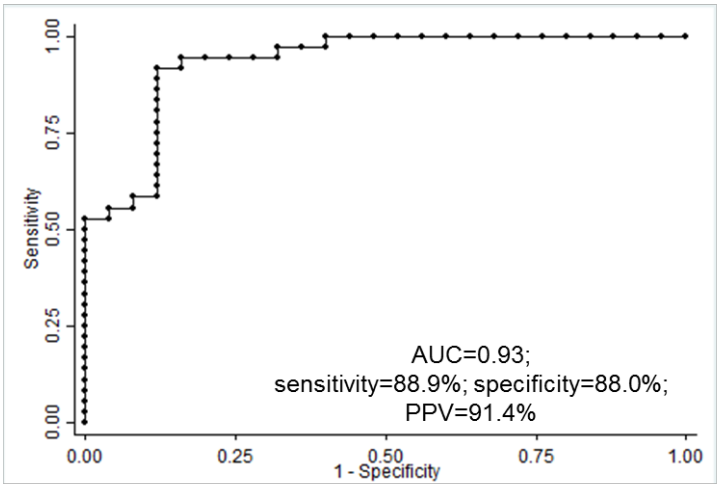
Variables: IHC frequency vs. pathology (cancer/benign)
Panel: CEP55, NUF2 and PBK

FIGURE 18 – ROC curve from multivariate logistic regression (MLR) for IHC frequency vs. pathology (cancer/benign). MLR was performed using the stepwise backwards logistic regression for pr=0.10. IHC intensity for three out of eight biomarkers (CEP55, NUF2 and PBK) resulted in a test with high sensitivity and specificity to discriminate cancer from benign tissue.



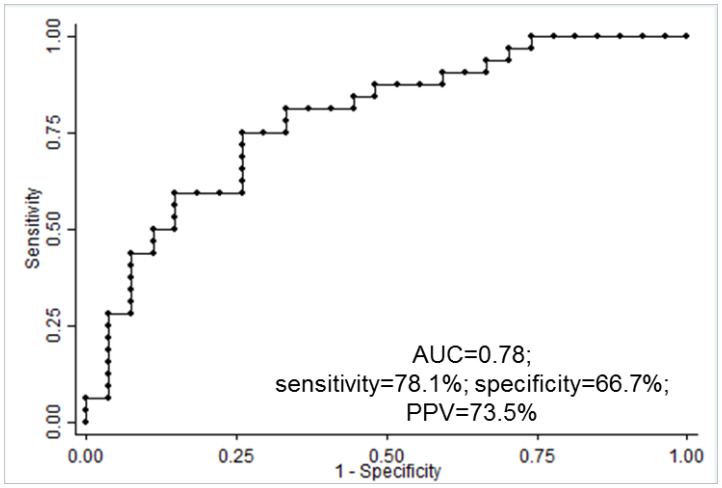
Variables: IHC intensity/frequency vs. pathology (cancer/benign)
Panel: CEP55, NUF2, PAGE4, PBK, RQCD1, SPAG4, SSX2 and TTK (intensity);
NUF2, PBK, SSX2 and TTK (frequency)

FIGURE 19 – ROC curve from multivariate logistic regression (MLR) for IHC intensity/frequency vs. pathology (cancer/benign). MLR was performed using the stepwise backwards logistic regression for pr=0.10. IHC intensity and/or frequency for CEP55, NUF2, PAGE4, PBK, RQCD1, SPAG4, SSX2 and TTK resulted in a test with high sensitivity and specificity to discriminate cancer from benign tissue.



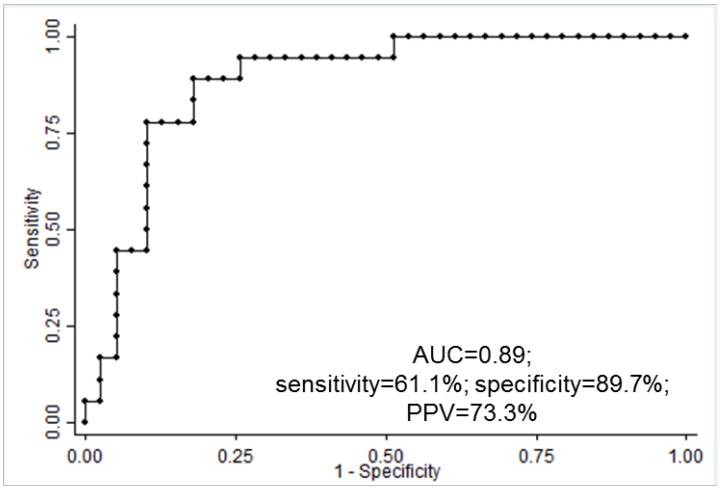
Variables: IHC vs. Gleason score (3+3/3+4 vs. 4+3/higher)
Panel: NUF2, PAGE4, PBK, RQCD1, and TTK (intensity);
PAGE4, RQCD1 and TTK (frequency)

FIGURE 20 – ROC curve from multivariate logistic regression (MLR) for IHC intensity/frequency vs. Gleason score (3+3/3+4 vs. 4+3/higher). MLR was performed using the stepwise backwards logistic regression for pr=0.20. IHC intensity and/or frequency for NUF2, PAGE4, PBK, RQCD1 and TTK resulted in a test with high sensitivity and specificity to discriminate cancer from benign tissue.



Variables: IHC vs. T stage (T1 + T2 vs. T3 + T4)
Panel: NUF2, PBK, SPAG4 and SSX2 (intensity);
CEP55, PBK and SSX2 (frequency)

FIGURE 21 – ROC curve from multivariate logistic regression (MLR) for IHC intensity/frequency vs. T stage (T1 + T2 vs. T3 + T4). MLR was performed using the stepwise backwards logistic regression for pr=0.30. IHC intensity and/or frequency for CEP55, NUF2, PBK, SPAG4 an SSX2 resulted in a test with high sensitivity and specificity to discriminate cancer from benign tissue.



Variables: IHC vs. PSA (≤10ng/mL vs. >10ng/mL)
Panel: CEP55, NUF2, RQCD1, SPAG4, SSX2 and TTK (intensity);
CEP55, NUF2, RQCD1 and TTK (frequency)

FIGURE 22 – ROC curve from multivariate logistic regression (MLR) for IHC intensity/frequency vs. PSA (≤10ng/mL vs. >10ng/mL). MLR was performed using the stepwise backwards logistic regression for pr=0.20. IHC intensity and/or frequency for CEP55, NUF2, RQCD1, SPAG4, SSX2 and TTK resulted in a test with high sensitivity and specificity to discriminate cancer from benign tissue.

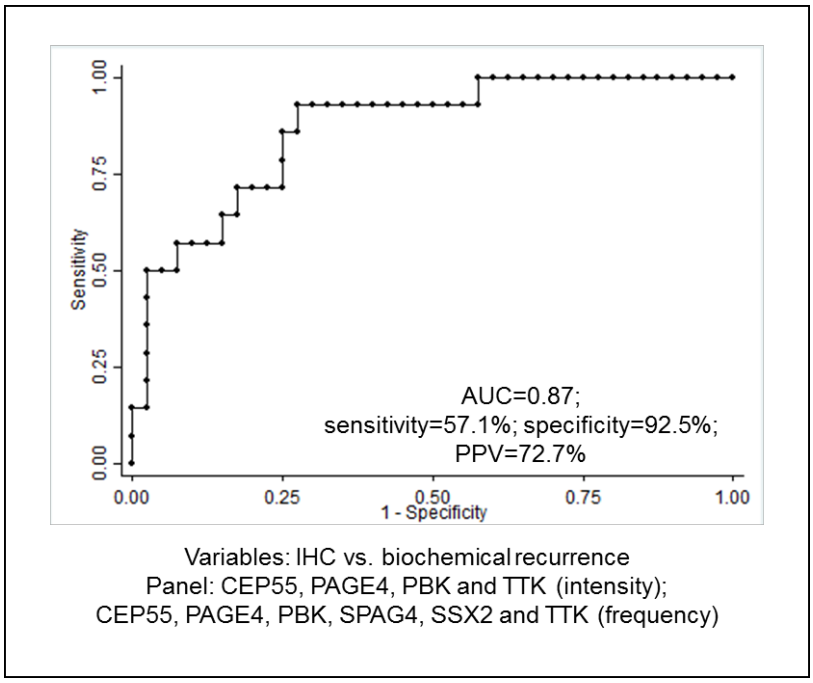


FIGURE 23 – ROC curve from multivariate logistic regression (MLR) for IHC intensity/frequency vs. biochemical recurrence. MLR was performed using the stepwise backwards logistic regression for pr=0.20. IHC intensity and/or frequency for CEP55, PAGE4, PBK, SPAG4, SSX2 and TTK resulted in a test with high sensitivity and specificity to discriminate cancer from benign tissue.

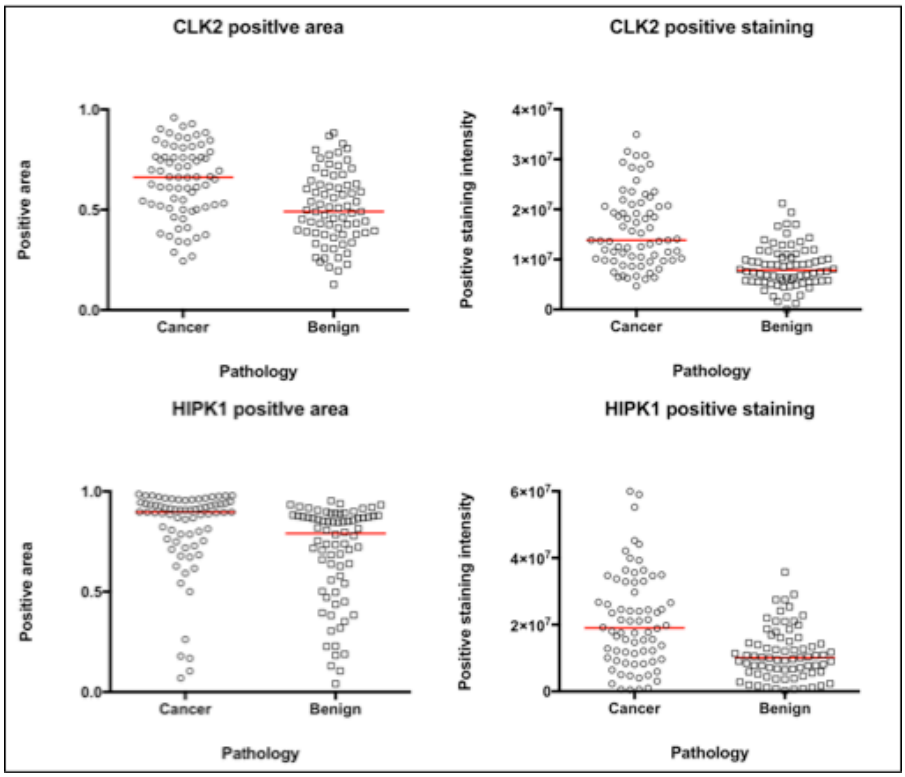


Figure 25 – CLK2 and HIPK1 expression in PCa cases and the paired benign adjacent tissue. Frequency (positive area) and intensity (positive staining) of IHC intensity were significantly higher in the tumor cases.

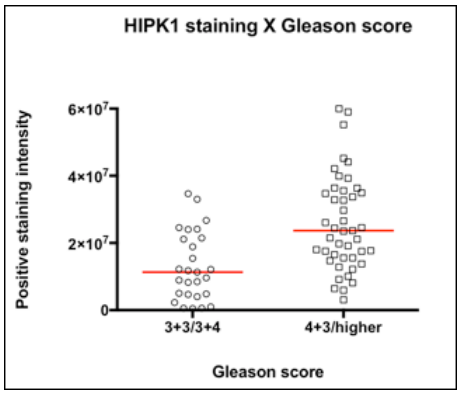


Figure 26 – HIPK1 expression in PCa cases according to Gleason score (3+3/ 3+4 vs. 4+3/higher). Expression of HIPK1 is significantly higher in more aggressive cases.